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FIRST TRIANNUAL REPORT (YEAR 2)

for period September 1, 1994 to December 31, 1994

Report Date: February 24, 1995

ONR Grant No. N00014-93-J-1034
(ECU Grant #5-01071)

PRECLINICAL INVESTIGATION OF LYOPHILIZED PLATELET PREPARATIONS

Principal Investigator: Arthur P. Bode, Ph.D.
East Carolina University
School of Medicine

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Attachments:

1. Report from subcontract principal investigator, Marjorie S. Read, Ph.D., The University of North Carolina at Chapel Hill.

2. Abstracts (3) from AABB and ASH. 1 - 4

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Administrative Activity:

Data from this project were presented in three abstracts at national meetings during this reporting period (see attached). Results from the testing of rehydrated platelets in the Clot Signature Analyzer and in the canine cardiopulmonary bypass model were given in a platform talk by the P.I. at the Annual Meeting of the American Association of Blood Banks (Nov. 12-17, 1994, San Diego, CA). At the American Society of Hematology meeting (Dec. 2-6, 1994, Nashville, TN), the P.I. presented a poster showing further studies of the rehydrated platelets in the Clot Signature Analyzer, and in the Blajchman thrombocytopenic rabbit model (project grant N00014-92-J-1244). Dr. Read also presented a poster on labelling of rehydrated platelets with radioactive or fluorescent tracers in preparation for circulatory life-span determinations of these platelets in vivo. Manuscripts expanding on these abstracts are in preparation.

Aim #5 was negatively impacted by loss of the ECU collaborating laboratory under Dr. Charles Knupp, due to severe illness and retirement of his technician. It was decided that further studies this year on SDS-PAGE analysis of biochemical signals of activation in rehydrated platelets were not feasible. A formal proposal for modification of the workplan will be submitted in the near future to ONR to redirect the resources that were earmarked for this effort. For the moment, other elements of Aim #5 will proceed, such as adhesion experiments and production of thromboxanes after stimulation of rehydrated platelets.

Downtime of the Virtis 600 Lyophilizer and Clot Signature Analyzer (CSA) at ECU has slowed progress on several aims. Virtis Corporation has now promised to overhaul our lyophilizer unit at no charge, and Xylum Corporation has apparently addressed the design problems we have experienced with the CSA needle punch assemblies. My laboratory at ECU will be relocated from the present double-wide trailer to floor space in the School of Medicine Brody Building in the next few weeks, probably causing more downtime which we will attempt to minimize by moving and setting up most of the lightweight equipment ourselves. For these and other reasons we will most likely have to petition for a no-cost extension of this grant later this year.

Scientific Progress:

The rest of this report details new results obtained at the ECU worksite. See attached subcontract report from the collaborating P.I. Dr. Read for results recently obtained at the University of North Carolina at Chapel Hill.

Since the last report (Year One Annual, up to Aug. 31, 1994), several more trials have been conducted in the canine cardiopulmonary bypass model for a total of 15 subject animals. The new data are consistent with our earlier observations that rehydrated canine platelets correct the prolonged bleeding time and abnormal CSA values caused by an acquired platelet function defect and thrombocytopenia in this model. Although these results seem complete upon initial review and received acceptance at their presentation at the AABB conference, it may prove necessary to perform a few more trials or controls to have a successful publication of these findings. Also, we are currently studying the possibility of adapting this model to

permit infusion and testing of human rehydrated platelets. If this appears to be feasible, a project modification proposal describing these new studies will be submitted as mentioned above.

Preparations are underway to perform initial studies in the second in vivo model of hemostasis involving hemostasis support of pigs after inducing a massive abdominal bleed by a tear in the aorta. We have begun making lyophilized platelet preparations from donor-pigs and have performed initial integrity studies on rehydrated aliquots of the final product. The protocol developed for stabilizing and lyophilizing human platelets was used without modification with success. Our first two trials of these preparations in the bleeding pig model were carried out in the time period between Dec. 31 and the date of this writing and will be presented in the next reporting period. We have prepared and stockpiled enough lyophilized pig platelets at this point to run 3 more trials, but will continue to make more pig platelet preparations whenever donor animals become available at our institution. Current plans are to perform at least 12 trials, with controls, over the next six months.

We have continued to perform analysis of hemostatic potential of rehydrated human, dog, or pig platelets in the CSA. In the period of this report, we tested 19 different platelet preparations along with an equal number of controls of fresh or stored blood bank platelets. In general the results have shown that the PARA21-type human lyophilized platelets show the best performance compared to PARA22 or PARA45-type platelets. The dog and pig rehydrated platelets have given variable results, which may have been due to interactions of plasma and cellular components in the reconstitution of whole blood for testing. We have found that pig or dog RBC cannot be used in combination with human plasma to test pig or dog platelet preparations; however, favorable results were obtained with pig or dog platelets resuspended in human plasma combined with washed human RBC. Under these conditions, the lyophilized platelet preparations from donor pigs and dogs have performed on the CSA in a manner comparable to fresh human platelets.

Future directions will include an emphasis on the in vivo testing of rehydrated platelets for hemostatic potential and circulatory lifespan. We hope that the CSA results will be predictive of in vivo performance so that this device could possibly become a sophisticated quality control monitor for our research preparations and for the pharmaceutical manufacture of human lyophilized platelets by Armour Pharmaceutical Corp.

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HEMOSTATIC PROPERTIES OF HUMAN LYOPHILIZED PLATELETS IN A THROMBOCYTOPENIC RABBIT MODEL AND A SIMULATED BLEEDING TIME DEVICE.

A. P. Bode, M. Blajchman, L. Bardossy*, and M.S. Read, Departments of Pathology, East Carolina University, Greenville, NC, and McMaster University, Hamilton, Ontario, and University of North Carolina at Chapel Hill, NC.

We have shown previously that our preparations of lyophilized human platelets (Lyo-Plt) are structurally intact upon reconstitution, and that they adhere to thrombogenic surfaces in vitro and in vivo (Blood 82:159a, 1993). Now we have tested the ability of these preparations to correct the ear bleeding time (BT) in rabbits made thrombocytopenic and immunosuppressed as detailed elsewhere (Blood 82:3489, 1993). Also, we have measured the in vitro bleeding time (IVBT) and collagen-induced thrombus formation (CITF) of Lyo-Plt in a prototype in vitro bleeding time device called the Clot Signature Analyzer (CSA, Xylum Corp, NY). In the rabbits, the endogenous platelet count was $\leq 10 \times 10^6$/mL and the BT was ≥ 900 seconds before infusion of 40-50 x 10^9 platelets. A platelet count and duplicate BTs were then performed one hour after infusion. For the CSA test, platelets were resuspended in fresh citrated plasma at 300-500 x 10^6/mL and combined with an equal volume of washed RBC to remake whole blood. $CaCl_2$ was added to 5 mM in the blood just prior to initiating each run. Mean results for Lyo-Plt versus human platelet-rich plasma (fresh) or 5-8 day old expired blood bank platelet concentrates (Exp PC) are tabulated below:

	Rabbit Model				Clot Signature Analyzer		
	n	% Recovery	Count	Ear BT	n	IVBT	CITF
Lyo-Plt	(12)	58%	108	234 sec.	(4)	118 sec.	73%
Fresh	(6)	79%	153	177 sec.	(4)	134 sec.	88%
Exp PC	(0)	ND	ND	ND	(3)	281 sec.	39%

The Lyo-Plt had reduced 1 hour recovery in the rabbit model relative to fresh platelets, but the mean BT result was similar to the value seen in non-infused rabbit controls with equivalent endogenous platelet counts. In the CSA, the Lyo-Plt gave results similar to that of fresh platelets and better than that of stored platelets in the IVBT and the CITF (t-test, $p < 0.05$). These findings support the notion that Lyo-Plts are hemostatically active.

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A STABLE RADIOLABEL FOR FRESH AND DRIED PLATELETS.

RJ Kowalsky, KK Taylor, DK McMahon, ME Brecher, DA Bellinger, RL Reddick, MS Read. University of North Carolina, Departments of Pathology, Pharmacy, and Radiology, Chapel Hill, NC.

In previous studies dried rehydrated platelets retained hemostatic properties and behaved like fresh platelets in animal models (PNAS submitted). However, dried platelets, unlike fresh platelets, did not retain a radiolabel. A stable radiolabel has been developed for fresh and stabilized lyophilized rehydrated canine platelets. ^{111}In-Tropolone, ^{51}Cr-Sodium Chromate, ^{125}I-NaI with Iodogen or Iodobeads, and ^{125}I-PKH95 were examined regarding labeling efficiency (LE), label stability over time in normal dog plasma or buffer, and botrocetin stimulation following radiolabeling. The table below indicates that ^{111}In-Tropolone and ^{125}I-PKH95 produced the highest LE. The high LE of ^{111}In-Tropolone, however, is compromised by poor label stability during incubation in NDP, with label translocation from platelets to plasma protein. The low LE and instability of ^{51}Cr and ^{125}I-NaI labels make them unsuitable platelet labels. Only ^{125}I-PKH95 provides satisfactory LE and label stability. Higher LE is achieved when mfr supplied buffer is used during labeling, however, this buffer causes aggregation of fresh platelets. Mfr buffer did not affect the LE, stability or botrocetin-induced aggregation of dried platelets. Substitution of PBS as the labeling buffer with ^{125}I-PKH95 lowered the LE but did not promote aggregation of fresh platelets during the labeling reaction. Both fresh and dried canine platelets labeled with ^{125}I-PKH95 in PBS demonstrate stable radiolabeled platelets that undergo botrocetin stimulated aggregation following labeling and warrants further consideration as a label for in vivo platelet survival studies.

Radiolabel	Percent LE Fresh	Percent LE Dried	Percent Bound Over Time * Fresh	Percent Bound Over Time * Dried
^{111}In-Trop	94	88	98 (16 hr) B	98 (16 hr) B
^{111}In-Trop	97	88	73 (20 hr) N	26 (6 hr) N
^{51}Cr-Sod Chr	19-22	0	28 (21 hr) N	--
^{125}I-Iodogen	2-9	19	46 (18 hr) N	48 (15 hr) B
^{125}I-Iodobeads	7-23	3	30 (18 hr) N	33 (5 hr) N
^{125}I-PKH95 (Mfr Buffer)	--	89-94	--	91 (22 hr) N 89 (72 hr) N
^{125}I-PKH95 (PBS)	67	65	93 (23 hr) N	97 (24 hr) N

* N = normal dog plasma @ 37°C B = ACD/Saline Buffer @ Rm Temp

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HEMOSTATIC PROPERTIES OF LYOPHILIZED PLATELETS IN TESTS OF BLEEDING TIMES.

Arthur P. Bode, Marjorie S. Read, Robert M. Lust. Depts. of Pathology and Surgery, East Carolina Univ., Greenville, NC, and Dept. Pathology, Univ. of N.C. at Chapel Hill.

Background: We have previously shown that lyophilized platelets (L-Plt) retain properties of adhesion and activatability in the Baumgartner perfusion chamber (Trans 33:72S, 1993). Now we have analyzed L-Plt in two systems directly testing hemostatic function. **Study Design:** One is a prototype device simulating the Ivy bleeding time in vitro (IVBT) and collagen-induced thrombus formation (CITF) in recalcified whole blood (Xylum Clot Signature Analyzer: CSA); the other is an in vivo bleeding time in dogs on full clinical heart-lung bypass before and after infusion of L-Plt. **Results:** On the CSA, L-Plt gave an average (n=4) IVBT of 1 min 58 sec and a CITF of 73% versus 2 min 14 sec and 88% respectively for fresh platelets. Expired platelet concentrates gave indeterminate results because aggregates clogged the lines. IVBT > 6 min and CITF < 25% is typical of vWD patients. In two canine heart-lung bypass studies, the in vivo bleeding time improved from >15 min to 5-7 min after infusion of a bolus of 2-3 x10^{11} L-Plt. The corrected count increments were (#1) 88% and (#2) 47% based on estimated circulatory volume. **Conclusions:** These results demonstrate the hemostatic activity of L-Plt and their potential value in transfusion medicine.

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September - December, 1994

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Performance Site: University of North Carolina at Chapel Hill
Principal Investigator: Marjorie S. Read, Ph. D.

1. Partial Characterization and Standardization of the Dried Platelet Product. (Specific Aim 1a)

Dense granules are a storage site for serotonin, ATP, ADP and calcium as well as other materials. Platelet dense granules will release their contents upon stimulation. In vitro, platelet uptake and release of labeled serotonin can be used as a measurement of platelet activity or platelet activation.

A fluorometric assay for serotonin detection has been adapted for the study of rehydrated platelets. Serotonin uptake and release is used to evaluate the ability of paraformaldehyde-treated platelets to undergo release, an energy driven response. We have compared the amount of serotonin secreted from fresh and paraformaldehyde treated platelets. We measured the amount of serotonin secreted from resting rehydrated platelets and compared it to the amount secreted by equal numbers of fresh platelets at 37°C. We made a similar study with thrombin-stimulated fresh and rehydrated platelets. A third study was performed to measure the amount of radiolabeled serotonin taken up by rehydrated platelets. Fresh platelets were capable of releasing serotonin at 37°C and at room temperature while rehydrated platelets secreted serotonin only at 37°C, with or without stimulation.

Our studies show that fresh platelets release approximately 0.5μm serotonin /10^{11} platelets after stimulation with thrombin, while rehydrated platelets release 0.2μm serotonin /10^{11} platelets (see Table 1). The reported amount of serotonin released from fresh platelets is 0.1 to 0.5μm serotonin /10^{11} platelets, suggesting that RP release of serotonin is within the normal range.

Table 1. Serotonin release from fresh and rehydrated platelets stimulated with thrombin.

[Thrombin] (U/ml)	Platelet Sample	Serotonin Released (μm/10^{11} platelets)
0.1	fresh	0.33
0.5	fresh	0.43
5.0	fresh	0.74
10.0	fresh	0.48
10.0	rehydrated	0.00
30.0	rehydrated	0.14
50.0	rehydrated	0.18
100.0	rehydrated	0.45

*normal values for fresh platelets are 0.1-0.5μm/10^{11} platelets with maximal stimulation

Note that the amout of thrombin needed for stimulation of rehydrated platelets is a 300-fold greater than that required for fresh platelets. Comparing these data to those seen in Table 2, we can see that thrombin stimulation is not necessary for serotonin release from rehydrated platelets when incubated at 37°C.

Table 2. Serotonin released from fresh and rehydrated platelets after incubation for 1 hour at 37°C without stimulation.

Platelet Sample	Serotonin Released ($\mu m/10^{11}$ platelets)
fresh	0.17
fresh	0.31
rehydrated	0.30
rehydrated	0.50

The ability of rehydrated platelets to take up serotonin was measured by incubation with ^{14}C-serotonin at 37°C. Rehydrated platelets (RP) appear to have minimal uptake of ^{14}C-serotonin as compared to fresh platelets. RPs take up 12% as much radiolabeled serotonin as fresh platelets and release 25% of the total amount taken up. Fresh platelets release 60% of the ^{14}C-serotonin initially taken up. No difference was seen in the amount of labeled serotonin present in platelets with the addition of serotonin release inhibitors . Uptake and release of serotonin did not appear to be dependent on temperature.

We are currently studying calcium ion transport and oxygen consumption of RP. We hope to learn more about the ability of platelets, which have been lightly treated with paraformaldehyde, to respond to stimuli and mimic fresh platelet responses.

2. Evaluation of the Drying Process (Specific Aim 1d).

We have been looking at the ability of the drying process to deliver a standard product. Transmission and Scanning electron microscopy (TEM and SEM) are being used to examine the morphology of different batches of RP. TEM allows us to determine degree of activation of RP as judged by pseudopod formation and centralization of granules. Measurements of the amount of spreading of RP compared to spreading of fresh platelets enables us to evaluate the ability of RP to spread on foreign and/or procoagulant surfaces. The retention of this adhesion/spread characteristic is esential for normal hemostais. RP prepared in our laboratory have so far shown some variability which can be attributed to the initial collection of blood and the storage time of platelet concentrate which we obtained from the blood bank, and not to the processing. There

has been little variability in the final product using the same starting material. These studies so far have emphasized the variability of fresh and stored platelets and not rehydrated platelets.

3. Evaluation of Paraformaldehyde as a Sterilizing Agent (Specific aim #3)

We have initiated studies on the bactericidal properties of the paraformaldehyde stabilization and lyophilization process. Platelet concentrates were obtained from the American Red Cross and prepared for lyophilization using sterile techniques. We have developed a process for preparing sterile preparation of RP. The paraformaldehyde treated preparations were rehydrated and aliquots of the sterile RP were removed and cultured over a period of 7 days for bacterial contamination. No growth was seen, indicating our handling techniques were adequate to produce a sterile paraformaldehyde-treated preparation. Following the development of a sterile method of preparation, experiments were performed to determine if the washing steps, the paraformaldehyde treatment, or the drying process contributed to or were sufficient for removal of bacteria from a contaminated platelet pack. Sterile platelet concentrates were inoculated with *Bacillus sereus* and the contaminated platelets were processed using the same sterile methods described above. The lyophilized platelet preparations were rehydrated and aliquots were removed and cultured for bacterial growth. Cultures were examined over a period of 7 days. We found no bacterial contamination in 6 of 6 platelet samples that we had inoculated.

To determine if the washing alone was sufficient to remove or reduce bacterial contamination we performed experiments in which platelets were inoculated with bacteria followed by washing in buffer but no paraformaldehyde treatment, while other contaminated preparations were were washed with buffer and treated with paraformaldehyde. All of the samples, which were washed but not treated with paraformaldehyde, cultured bacteria within 48 hours of plating. None of the paraformaldehyde treated samples grew bacteria. It appears evident that paraformaldehyde, even in the low concentrations and limited exposure time used in our procedure, is an effective bactericidal agent. Similar studies are underway with other commonly seen agents such as *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* to determine if paraformaldehyde acts as a bactericidal agent for a broad range of bacteria. See Table 3 on the next page for a compilation of these data.

Table 3. Bactericidal effects of paraformaldehyde in the stabilization and lyophilization process.

sample number	inoculated bacteria	paraformaldehyde treatment	bacterial growth
1	*B. sereus*	None	+
2	*B. sereus*	None	+
3	*B. sereus*	None	+
4	*B. sereus*	None	+
5	*B. sereus*	None	+
6	*B. sereus*	None	+
7	*B. sereus*	+	None
8	*B. sereus*	+	None
9	*B. sereus*	+	None
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